ISOLATING A CYTOPLASMIC FRACTION WITHOUT IMPAIRING THE VIABILITY OF OOCYTES AND EMBRYONIC CELLS

TECHNICAL FIELD

The present invention relates to a method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized. The invention also relates to a method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell.

BACKGROUND ART

Methods for the analysis of the oocyte cytoplasm have necessitated the destruction of the oocyte. Consequently, although the information retrieved in the analysis may be of particular interest, the investigator is unable to further study the oocyte as a complete cellular unit in the context of the information retrieved from the analysis. Further, the oocyte cannot subsequently be fertilized and allowed to develop into an embryo, fetus and infant.

There is a need for a method which allows the study of cytoplasmic mechanisms in the oocyte which does not impair the capacity of the oocyte to be fertilized. There is also a need for a method which allows the study of cytoplasmic mechanisms in an embryonic cell which does not impair the developmental potential of the cell. These methods would be useful for studying those mechanisms in an oocyte or embryonic cell which are mediated by mictochondria and which are the cause of, or are suspected of causing, or are associated with, dysfunction or disease, either in the oocyte or embryonic cell, or in progeny descended from the fertilized oocyte, or the embryonic cell.

DESCRIPTION OF THE INVENTION

The present invention seeks to address these needs and in a first aspect provides a method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the step of releasing a cytoplasmic fraction from the oocyte. The volume of cytoplasmic fraction which is released from the oocyte is about 5% of the volume of the oocyte. Preferably the volume is about 2% of the volume of the oocyte.

In an embodiment of the first aspect of the invention, the method includes the following steps:

a) inserting releasing means into the oocyte;

b) drawing a cytoplasmic fraction which is about 5% of the volume of the oocyte into the releasing means; and

c) withdrawing the releasing means from the oocyte so that the fraction is isolated in the releasing means.

The volume of the cytoplasmic fraction drawn into the releasing means is typically less than 10 pL and preferably 8 pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100μm into the pipette.

The drawing of the cytoplasmic fraction from the oocyte into the releasing means typically forms an extrusion of cytoplasmic contents between the releasing means and the oocyte. The cytoplasmic fraction is isolated from the oocyte by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

Typically, the cytoplasmic fraction contains cytoplasmic organelles, and includes or consists of a sample of the oocyte's mitochondria and mitochondrial products.

Mitochondria located in an oocyte are a template from which all mitochondria in the progeny descended from the fertilized oocyte are derived. The present inventors recognised that a method for isolating a cytoplasmic fraction from an oocyte, without impairing the capacity of the oocyte to be fertilized, would allow the study of the relationship between the integrity of the mitochondrial genome and the function of both the oocyte and the progeny descended from the fertilized oocyte.

Thus, in a second aspect, the invention relates to a method of analysing the mitochondrial genome of mitochondria located in an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) analysing the mitochondrial genome of the mitochondria in the fraction.

Mutation of the mitochondrial genome causes, or at least is associated with, dysfunction or disease. For example, the nucleotide sequence deletion from nucleotide position number 8470 to 13,446 of the mitochondrial genome, the so called "5kb common deletion", is understood to be associated with Kearns-Sayre syndrome (KSS) and chronic progressive external opthalmoplegia (CPEO). Other disease causing deletions, which may or may not be observed with the common deletion, include a 7.4 kb deletion and 10.4 kb deletion/insertion in the

mitochondrial genome of brain and heart, as well as various point mutations. The deletion is also observed in human tissue including skeletal muscle, heart, brain, oocytes, leukocytes, retina and ovaries.

Over 40 pathogenic point mutations of the mitochondrial genome can be associated with a broad spectrum of degenerative diseases involving the central nervous system, heart, muscle, endocrine system, kidney and liver. Diseases associated with point mutations include Leigh Syndrome, MELAS (mitochonrdial encephalomyopathy, lactic acidosis and stroke like episodes), MERRF (myoclonus epilepsy with ragged-red fibres), NARP (neruopathy, ataxia and retinitis pigmentosa) and LHON (Leber hereditary optic neuropathy).

A method of isolating a cytoplasmic fraction from an oocyte without impairing the capacity of the oocyte to be fertilized is useful for detecting nucleotide sequence mutations in the mitochondrial genome, enabling the study of a nucleotide sequence, polymorphism or mutation in the context of the functional integrity of both the oocyte and the progeny descended from the fertilized oocyte.

Thus, in a third aspect, the invention provides a method of detecting a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of mitochondria located in an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention; and

b) analysing the nucleotide sequence of the mitochondrial genome of the mitochondria in the cytoplasmic fraction for the presence of a

nucleotide sequence, polymorphism or mutation in the mitochondrial genome.

As mitochondrial genome mutations which cause, or are associated with, disease or dysfunction, including point mutations, are almost exclusively maternally inherited, a method of isolating a cytoplasmic fraction without impairing the capacity of the oocyte to be fertilized is useful for predicting whether the progeny descended from a fertilized oocyte will or will not contain a nucleotide sequence, polymorphism or mutation of the mitochondrial genome which causes, or is suspected of causing, or is associated with, disease or dysfunction.

Thus in a fourth aspect, the invention provides a method for predicting whether the progeny descended from a fertilized oocyte will contain a nucleotide sequence, polymorphism or mutation in a mitochondrial genome which causes, or is suspected of causing, or is associated with, a disease or dysfunction, wherein the method does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention; and

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the nucleotide sequence, polymorphism or mutation in the mitochondrial genome;

wherein the presence of the nucleotide sequence, polymorphism or mutation indicates a likelihood that the progeny descended from the fertilized oocyte will contain the nucleotide sequence, polymorphism or mutation.

The mere existence of a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of

mitochondria in an oocyte, which is known to be associated with, or cause, or is suspected of causing, disease or dysfunction, may not be sufficient to mediate the disease or dysfunction in the oocyte itself, or the progeny descended from the fertilized oocyte. Indeed, there appears to be at least an additional factor which contributes to the likelihood of, and/or the severity of the disease or dysfunction in the oocyte containing the nucleotide sequence, polymorphism or mutation, or the progeny descended from the fertilized oocyte. That is, it is likely that the actual proportion, or "threshold level", of mitochondria which contain the particular nucleotide sequence, polymorphism or mutation in the mitochondrial genome in the oocyte will contribute to the likelihood and/or severity of disease or dysfunction in the oocyte, or progeny descended from the fertilized oocyte. In particular, and in contrast with the nuclear genome, there are of the order of at least 100,000 copies of a mitochondrial genome in an oocyte. "Heteroplasmy" is observed when an oocyte contains more than one species of mitochondrial genome. An oocyte, and indeed the tissues of the progeny descended from the fertilized oocyte, become "heteroplasmic" when a nucleotide sequence, polymorphism or mutation is introduced into a cell which, before the introduction, contained a single species of mitochondrial genome. With regard to the nucleotide sequences, polymorphisms and mutations of the mitochondrial genome which cause, or are suspected of causing, or are associated with, a disease or dysfunction, it is generally recognised that when a particular level of heteroplasmy is surpassed, the manifestations of the disease or dysfunction are sooner or later observed.

Based on the observation that mitochondria in the mouse oocyte migrate to specific regions of the oocyte cytoplasm, in particular, the perinuclear region, at specific stages of meiosis, to obtain a sample which is representative of all mitochondrial genomes in the human oocyte, a method for determining the degree of heteroplasmy in a human oocyte is typically applied at specific stages of meiosis. A method of isolating cytoplasmic fractions which does not impair the capacity of the oocyte to be fertilized is useful for determining the level of heteroplasmy in the oocyte, and for predicting the average level or likely range of heteroplasmy in tissues of the progeny descended from the fertilized oocyte.

Thus, in a fifth aspect, the invention provides a method of determining the level of heteroplasmy of mitochondrial genomes in an oocyte which does not impair the capacity of the oocyte to be fertilized, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) comparing the number of mitochondrial genomes in the fraction with a nucleotide sequence, polymorphism or mutation, with the number of genomes without the nucleotide sequence, polymorphism or mutation in the fraction.

As the level of heteroplasmy and the existence of a nucleotide sequence, polymorphism or mutation which causes, or is suspected of causing, or is associated with, disease or dysfunction, in progeny descended from a fertilized oocyte contribute to the likelihood and/or severity of disease or dysfunction, the method of

determining the level of heteroplasmy of mitochondrial genomes in an oocyte is useful for predicting whether the progeny descended from the fertilized oocyte are likely to suffer from a disease or dysfunction which is caused by, or associated with, the particular nucleotide sequence, polymorphism or mutation, and/or the severity of the disease or dysfunction.

Thus in a sixth aspect, the invention provides a method of determining whether the progeny descended from a fertilized oocyte are likely to suffer from a disease or dysfunction caused by, or suspected of being caused by, or associated with, a nucleotide sequence, polymorphism or mutation in a mitochondrial genome, wherein the method does not impair the capacity of the oocyte to be fertilized and includes the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the nucleotide sequence, polymorphism or mutation; and

determining that the progeny are likely to suffer from the disease or dysfunction where the analysis of the mitochondrial genome of mitochondria in the fraction demonstrates that the level of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation in the oocyte is at least the same as the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

As noted above, methods for the analysis of cytoplasmic fractions from an oocyte involve the

destruction of the oocyte. Although these methods reveal useful information about the oocyte that is thereby destroyed, the value of this information is confined to an extrapolation to other oocytes which are not evaluated. As a population of oocytes from the same individual are typically heterogenous with respect to ooplasmic content, there is some question as to the extent to which the information derived from the oocyte which is destroyed can be accurately or reliably extrapolated to other oocytes. That is, using these methods one cannot conclude that because an oocyte is found to have either no nucleotide sequence, polymorphism or mutation in the mitochondrial genome which causes, or is associated with, disease or dysfunction in the oocyte, or the progeny of the fertilized oocyte, or a low degree of heteroplasmy in relation to the nucleotide sequence, polymorphsim or mutation, that other oocytes derived from the same individual will have the same mitochondrial genotype. Consequently, these methods for the analysis of oocytes are of limited use in the field of *in vitro* fertilization, where it is anticipated that some oocytes derived from a patient may contain a nucleotide sequence, polymorphism or mutation which causes, or is suspected of causing, or is associated with, disease or dysfunction in the progeny descended from the fertilized oocyte.

The method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to be fertilized is particularly useful for screening oocytes for the presence of a nucleotide sequence, polymorphism or mutation which causes, or is suspected of causing, or is associated with, disease or dysfunction in the progeny descended from the fertilized oocyte, prior to fertilization.

Thus in a seventh aspect, the invention provides a method of screening an oocyte for the presence of a nucleotide sequence, polymorphism or mutation in the mitochondrial genome in the oocyte which causes, or is suspected of causing, or is associated with, disease or dysfunction in the progeny descended from the fertilized oocyte, wherein the method does not impair the capacity of the oocyte to be fertilized, and includes the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the sequence, polymorphism or mutation.

Patients who present for *in vitro* fertilization treatment frequently have few oocytes available for selection for fertilization, and of the available oocytes, it is anticipated that some of these will contain a nucleotide sequence, polymorphism or mutation in a mitochondrial genome which causes or is associated with a disease or dysfunction in progeny descended from the fertilized oocyte. Other methods for isolating a cytoplasmic fraction from an oocyte are particularly unsuitable for selecting oocytes for fertilization, and could potentially result in the destruction of oocytes which do not contain a nucleotide sequence, polymorphism or mutation in a mitochondrial genome which causes or is associated with a disease or dysfunction, or oocytes which have a low level of heteroplasmy with respect to that mutation.

The method of isolating a cytoplasmic fraction from an oocyte which does not impair the capacity of the oocyte to

be fertilized is particularly useful for selecting oocytes for fertilization which do not contain a nucleotide sequence, polymorphism or mutation which causes, or is suspected of causing, or is associated with, disease or dysfunction in the progeny descended from the fertilized oocyte, prior to fertilization.

Thus in an eighth aspect, the invention relates to a method of selecting an oocyte for fertilization which either:

(i) does not contain a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of mitochondria in the oocyte which causes, or is suspected of causing, or is associated with, a disease or dysfunction in the progeny descended from the fertilized oocyte; or

(ii) has a level of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation which is less than the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction;

wherein the method does not impair the capacity of the oocyte to be fertilized, and includes the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) analysing the mitochondrial genome of the mitochondria in the fraction; and

c) selecting the oocyte for fertilization, provided that at least the degree of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation in the mitochondrial genome of the oocyte, is less

than the degree of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

The oocytes which are screened or selected in accordance with the seventh or eighth aspect of the invention, respectively, may be fertilized by intra cytoplasmic sperm injection (ICSI), or by *in vitro* fertilization. Preferably the oocytes are fertilized by ICSI.

To the extent that the eighth aspect of the invention is particularly suitable for selecting oocytes for fertilization and can be used prior to ICSI or IVF, the present inventors recognise that the method embodied in the eighth aspect of the invention is an integral part of a novel *in vitro* fertilization procedure.

Accordingly, in a ninth aspect, the invention provides a method of fertilizing an oocyte, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the oocyte according to the method of the first aspect of the invention;

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of a nucleotide sequence, polymorphism or mutation which causes, or is suspected of causing, or is associated with, a disease or dysfunction in progeny descended from the fertilized oocyte; and

c) fertilizing the oocyte, provided that the degree of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation in the oocyte, is less than the degree of heteroplasmy which is known to be

associated with the manifestation of the disease or dysfunction.

The mitochondrial genome of mitochondria which are in the cytoplasmic fraction isolated according to the method of the first aspect of the invention can be analysed according to standard techniques. These techniques are exemplified further herein and include the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, genomic hybridisation, nucleotide sequencing and gene function detection and/or measurement.

When determining the level of heteroplasmy of mitochondrial genomes in the oocyte, a representative fraction of genomes can be obtained when mitochondria are randomly distributed in the cytoplasm. Preferably a cytoplasmic fraction is drawn from the oocyte when the distribution of mitochondria in the oocyte can be expected to be random, including for example at the germinal vesicle (GV) stage of the primary oocyte and /or at a stage from the metaphase II stage of meiosis of the secondary oocyte, to prior to syngamy.

As the method of isolating a cytoplasmic fraction from an oocyte does not interfere significantly with the metabolism of the oocyte, the present inventors recognised that the method of isolating a cytoplasmic fraction is useful for studying the cytoplasm of other cells, for example embryonic cells, without impairing the developmental potential of these cells.

Thus, in an tenth aspect, the invention provides a method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell, the method including the step of releasing a cytoplasmic fraction from the cell. The

volume of cytoplasmic fraction which is released from the cell is about 5% of the volume of the cell. Preferably the volume is about 2% of the volume of the cell.

In an embodiment of the tenth aspect of the invention, the method includes the steps of:

a) inserting releasing means into the embryonic cell;

b) drawing a cytoplasmic fraction which is about 5% of the volume of the embryonic cell into the releasing means; and

c) withdrawing the releasing means from the cell so that the fraction is isolated in the releasing means.

The volume of the cytoplasmic fraction drawn into the releasing means is typically less than 10pL and preferably 8pL. Preferably, the releasing means includes at least an ICSI pipette and preferably the cytoplasmic fraction is drawn approximately 100µm into the pipette.

The drawing of the cytoplasmic fraction from the embryonic cell into the releasing means typically forms an extrusion of cytoplasmic contents between the releasing means and the embryonic cell. The cytoplasmic fraction is isolated from the embryonic cell by gently stretching or shearing the extrusion so as to separate the extrusion. Preferably the cytoplasmic fraction is isolated by stretching the extrusion.

Typically, the cytoplasmic fraction contains cytoplasmic organelles, and includes or consists of a sample of the mitochondria and mitochondrial products of the embryonic cell.

As described herein above, various deletions and point mutations of the mitochondrial genome are known to cause, or at least to be associated with, dysfunction or disease.

A method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell is useful for detecting a nucleotide sequence, polymorphism or mutation in the mitochondrial genome, enabling the study of the nucleotide sequence, polymorphism or mutation in the context of the functional integrity of both the embryonic cell and the progeny descended from the embryonic cell.

Thus in an eleventh aspect, the invention provides a method of detecting a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of mitochondria located in an embryonic cell which does not impair the developmental potential of the cell, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the embryonic cell according to the method of the tenth aspect of the invention; and

b) analysing the nucleotide sequence of the mitochondrial genome of the mitochondria in the cytoplasmic fraction for the presence of a nucleotide sequence, polymorphism or mutation in the mitochondrial genome.

The present inventors recognised that a method of isolating a cytoplasmic fraction without impairing the developmental potential of an embryonic cell is useful for predicting whether the progeny descended from the embryonic cell will or will not contain a nucleotide sequence, polymorphism or mutation of the mitochondrial genome which causes, or is suspected of causing, or is associated, with disease or dysfunction.

Thus, in a twelfth aspect, the invention provides a method for predicting whether the progeny descended from

an embryonic cell will contain a nucleotide sequence, polymorphism or mutation in a mitochondrial genome which causes, or is suspected of causing, or is associated with, a disease or dysfunction, wherein the method does not impair the developmental potential of the cell, the method including the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the embryonic cell according to the method of the tenth aspect of the invention; and

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the nucleotide sequence, polymorphism or mutation;

wherein the presence of the nucleotide sequence, polymorphism or mutation indicates a likelihood that the progeny descended from the embryonic cell will contain the nucleotide sequence, polymorphism or mutation.

As discussed herein above, it is likely that a particular level of heteroplasmy must be surpassed before the manifestations of disease or dysfunction are observed, which are caused by, or associated with, a nucleotide sequence, polymorphism or mutation of the mitochondrial genome.

Thus, in a thirteenth aspect, the invention provides a method of determining the level of heteroplasmy of mitochondrial genomes in an embryonic cell which does not impair the developmental potential of the cell, and which includes the following steps

a) isolating a cytoplasmic fraction which includes mitochondria from the embryonic cell according to the method of the tenth aspect of the invention; and

b) comparing the number of mitochondrial genomes in the fraction with a nucleotide sequence,

polymorphism or mutation, with the number of genomes without the nucleotide sequence, polymorphism or mutation in the fraction.

In a fourteenth aspect, the invention provides a method of determining whether the progeny descended from an embryonic cell are likely to suffer from a disease or dysfunction caused by, or suspected of being caused by or associated with, a nucleotide sequence, polymorphism or mutation in a mitochondrial genome, wherein the method does not impair the developmental potential of the cell, and includes the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the embryonic cell according to the method of the tenth aspect of the invention; and

b) analysing the mitochondrial genome of the mitochondria in the fraction for the presence of the nucleotide sequence, polymorphism or mutation;

determining that the progeny are likely to suffer from the disease or dysfunction where the analysis of the mitochondrial genome of mitochondria in the fraction demonstrates that the level of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation in the embryonic cell is at least the same as the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

Prior to commencing the embryo transfer procedure it is important to select

(i) an embryo which does not contain a nucleotide sequence, polymorphism or mutation of the mitochondrial genome which causes, or is suspected of causing, or is associated with, disease or dysfunction, and/or

(ii) an embryo which has a low level of heteroplasmy with respect to that nucleotide sequence, polymorphism or mutation.

The method of isolating a cytoplasmic fraction from an embryonic cell which does not impair the developmental potential of the cell is particularly useful for selecting an embryo for embryo transfer which does not contain a nucleotide sequence, polymorphism or mutation which causes or is associated with disease or dysfunction in the progeny descended from the embryonic cell, prior to embryo transfer.

Thus, in a fifteenth aspect, the invention provides a method of selecting an embryo for embryo transfer, which either:

(i) does not contain a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of mitochondria in an embryonic cell derived from the embryo, which causes, or is suspected of causing, or is associated with, a disease or dysfunction in the progeny descended from the embryonic cell or embryo; or

(ii) has a level of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation which is less than the level of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction;

wherein the method does not impair the developmental potential of the embryo, and includes the following steps:

a) isolating a cytoplasmic fraction which includes mitochondria from the embryonic cell according to the method of the tenth aspect of the invention; and

b) analysing the mitochondrial genome of the mitochondria in the fraction; and

c) selecting the embryo for embryo transfer, provided that at least the degree of heteroplasmy of mitochondrial genomes with respect to the nucleotide sequence, polymorphism or mutation in the mitochondrial genome of the embryonic cell, is less than the degree of heteroplasmy which is known to be associated with the manifestation of the disease or dysfunction.

In a seventeenth aspect, the invention relates to a kit for use in the method of isolating a cytoplasmic fraction from an oocyte, or from an embryonic cell. The kit includes at least one nucleotide probe specific for a nucleotide sequence, polymorphism or mutation in the mitochondrial genome of mitochondria in an oocyte or an embryonic cell, which causes, or is suspected of causing, or which is associated with a disease or dysfunction in the progeny descended from the fertilized oocyte or embryonic cell. In one embodiment, the at least one nucleotide probe is specific for any one of the nucleotide sequence mutations in the mitochondrial genome shown in Table 1.

Table 1

Position	RNA	Gene	Disease
721	r	12s	ADPD Alzheimers/Parkinsons
1555	r	12s	DEAF, *weak*
1606	t	Val	Encephalomyopathy
1642	t	Val	MELAS
1644	t	Val	Leighs
3010	r	16s	longevity
3196	r	16s	ADPD
3243	t	Leu(UUR)	MELAS
3250	t	Leu(UUR)	MM Mitochondrial myopathy
3251	t	Leu(UUR)	MM
3254	t	Leu(UUR)	diabetes
3256	t	Leu(UUR)	
3260	t	Leu(UUR)	HCM Hypertrophic cardiomyopathy
3271	t	Leu(UUR)	MELAS *later onset than 3243*
3288	t	Leu(UUR)	MM
3302	t	Leu(UUR)	MM
3303	t	Leu(UUR)	HCM/MM
3394	m	ND1	LHON also implicated in long U interval in ECG
3397	m	ND1	ADPD
3460	m	ND1	LHON *severe*
4160	m	ND1	LHON+
4216	m	ND1	LHON
4269	t	Ile	FICP Fatal infantile cardiomyopathy plus
4274	t	Ile	CPEO
4317	t	Ile	FICP
4336	t	Gln	ADPD (*weak*)
4917	m	ND2	LHON
5178	m	ND2	longevity
5244	m	ND2	LHON

Position	RNA	Gene	Disease	
5521	t	Trp	Myopathy, late-onset	
6480	m	COI		
6930		COI	G6930A (stop codon):multisystem	
7444	m	COI	LHON	
7445	m	COI	PPK	Palmoplantar keratoderma & hearing loss
7472	t	Ser		Myoclonus epilepsy, w/o RRF
7497	t	Ser	MERRF	Myoclonus epilepsy etc
7512	t	Ser		Myoclonus epilepsy, w/o RRF
8342	t	Lys		Ext. ophthalmoplegia, myopathy, w.o RRF
8344	t	Lys	MERRF	Myoclonic epilepsy, ragged red fibres; lipomatosis
8356	t	Lys	MERRF	
8414	m	ATP8	longevity	
8851		ATP6		Bilateral striatal necrosis
8860		ATP6	normal	A->G
8993	m	ATP6	NARP/Leighs	Neurogenic weakness, ataxia, retinitis
9176	m	ATP6		Bilateral striatal necrosis
9438	m	COI	LHON	
9804	m	COI	LHON	

Position	RNA	Gene	Disease
9997	t	Gly	HCM
10004	t	Gly	Sudden childhood death?
10010	t		encephalomyopathy
10410	t	Arg	Alpers syndrome
11778	m	ND4	LHON *severe*
18832	m	ND4	myopathy
12258	t	Leu2 (CUN)	retinitis pigmentosa, sensorineural hearing loss
12300	t	Leu2 (CUN)	Mutation of anticodon protects against UUR reading
12320	t	Leu2 (CUN)	Increased in age in one patient with myopathy
13708	m	ND5	LHON
13730	m	ND5	LHON
14459	m	ND6	LHON or Leighs
14569	m	ND6	LHON or Leighs
14484	m	ND6	LHON *severe*
14709	t	Glu	Varied: infantile myopathy to NIDDM
14826		cyt b	exercise intolerance
15084	m	cyt b	exercise intolerance
15168	m	cyt b	exercise intolerance
15257	m	cyt b	LHON
15498	24nt del	cyt b	exercise intolerance
15723	m	cyt b	exercise intolerance
15762	m	cyt b	Myopathy, late-onset
15812	m	cyt b	LHON
15923	t	Thr	LIMM Lethal infantile mitochondrial myopathy

Position	RNA	Gene	Disease
15990	t	Pro	MM
16189	D	NIDDM	Non-insulin dependent diabetes

N= any nucleotide

R= either puRine (A,G)

Y= either pYrimidine (U,C)

The mitochondrial genome of mitochondria which are in the cytoplasmic fraction isolated according to the method of the tenth aspect of the invention can be analysed according to standard techniques. These techniques are exemplified further herein and include the polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis, genomic hybridisation, nucleotide sequencing and gene function detection and/or measurement.

In another aspect, the invention provides nucleotides including the following sequences:

ATP6F: TCACCACCCAACAATGAC

ATP6R: TAAGGCGACAGCGATTTC.

DEFINITIONS:

In the specification and claims, "oocyte" means a female germ line cell including primary oocytes and secondary oocytes, and includes human oocytes. Primary oocytes include germ cells at the GV (germinal vesicle) stage of meiosis. Secondary oocytes include germ cells at the metaphase II stage of meiosis.

In the specification and claims, "progeny descended from a fertilized oocyte" means the individual which is generated from the fertilization of the female germ cell with the male germ cell. "Individual" means the multicellular organism from the earliest stage of

embryonic life (for example, the 2 cell stage) to adult life.

In the specification and claims, "does not impair the capacity of the oocyte to be fertilized" means that the oocyte which has had a fraction of cytoplasm isolated, may be fertilized, or in other words, may undergo any one or more of the biochemical or cellular events which are associated with any one or more of the stages of fertilization, from the activation of the oocyte by entry of sperm, to the generation of a zygote and the formation of cleavage products of the zygote. Typically after the fraction of the cytoplasm is isolated, the oocyte may be fertilized in vitro by standard techniques, including for example ICSI and IVF.

In the specification and claims, "embryonic cell" includes a post-syngamous fusion product of the female and male germ cells, a zygote, and cleavage products of a zygote at any stage of development from the 2 cell stage to the stage of implantation.

In the specification and claims, "progeny descended from an embryonic cell" or "progeny descended from an embryo" means the individual which is generated from the post syngamous fusion product of the female and male germ cells. "Individual" means the multi-cellular organism from the earliest stage of embryonic life (for example, the 2 cell stage) to adult life.

In the specification and claims "does not impair the developmental potential of the cell" or "does not impair the developmental potential of the embryo" means that the embryonic cell which has had a fraction of cytoplasm isolated, and the embryo from which the embryonic cell may be derived, may undergo any one or more of the biochemical

or cellular events which are associated with cell differentiation and/or maturation.

BRIEF DESCRIPTION OF FIGURES

Figure 1 shows a 400 by fragment amplified from the D-loop region of the mitochondrial genome derived from cytoplasmic biopsy samples of oocytes A1 to A7, using oligonucleotide primers L29 and H04.

Figure 2 shows amplification of a 271 bp fragment of mitochondrial genomes isolated from embryonic cells using primers corresponding to mtDNA positions 8201 and 8472..

BEST METHOD FOR CARRYING OUT THE INVENTION

1. BIOPSY of OOCYTES

MATERIALS AND METHODS

Source of oocytes

Human oocytes A1 to A5 were donated for research. These oocytes were either germinal vesicle (GV) cells or were at the MI stage of meiosis, and were 6 hours postretrieval.

Human oocytes B1 to B8 were donated for research. These oocytes were at the MII stage of meiosis and were 24 hours post-retrieval.

Human oocyte C1 was designated at the patients request to be part of the study. This oocyte was at the MII stage of meiosis and was 24 hours post-retrieval.

Isolation of oocyte cytoplasmic fractions

A small amount of ooplasmic material was biopsied from human oocytes using an ICSI pipette (Sydney IVF, Sydney). Briefly, the pipette is a glass capillary drawn out to have an end diameter of approximately 7µm with a bevelled tip.

The biopsy technique was performed as follows: ooplasm was drawn into the pipette to a distance of

approximately 100µm (approximately 8pl). The pipette was then withdrawn from the oocyte, forming a thin ooplasmic bridge, which was then broken by stretching. Each ooplasmic biopsy was expelled directly into a PCR tube containing 20µlof PCR buffer, Proteinase K and 20 mM DTT. Tubes were incubated either at 37°C overnight, or at 50°C for 30 minutes and then frozen. Both protocols were followed with heat inactivation of Proteinase K at 95°C for 10 minutes.

Analysis of mitochondrial genome in oocyte cytoplasmic fractions

The mitochondrial genome in the oocyte cytoplasmic fractions was analysed by the polymerase chain reaction (PCR).

(i) D-loop fragment

Reactions of 20µl were established; 10 µl of reaction mixture was added to 10µl of ooplasmic biopsy preparation. The 101 reaction mixture contained 2.5 pmol of each primer, 200µM of each dNTP, PCR buffer, milli-Q water and 0.5 units of Taq. All reactions were carried out in capped 0.2ml tube strips. PCR cycling was performed in an FTS Thermal Sequencer (Corbett Research, Sydney, NSW) under the following conditions: initial denaturation at 93°C for 5 minutes, followed by 24 to 40 cycles of 93°C denaturation for 45 seconds, 60°C annealing for 1 minute and 72°C extension for 1 minute; ending with a polishing step of 72°C for 7 minutes, cooling to 15°C and holding at 4°C. Complete reaction mixtures lacking template DNA were included in all PCR reactions as negative controls.

Primers L29 (5'- GGTCTATCACCCTATTAACCAC-3') and H04 (5'-CTGTTAAAAGTGCATACCGCCA-3'), specific for a 400bp sequence in the mitochondrial D-loop region were used.

(ii) Common deletion fragment

An identical protocol to that described above was used, but with the following conditions: initial denaturation at 95°C for 3 minutes followed by 20 to 35 cycles of 92°C denaturation for 1 minute, 60°C annealing for 10 seconds and 68°C extension for 45 seconds; ending with a polishing step of 75°C for 7 minutes, cooling to 15°C and holding at 4°C. From cycle 11, 15 seconds was added to the extension time every cycle. Complete reaction mixtures lacking template DNA were included in all PCR reactions as negative controls.

Primers L820 (5'-TTCATGCCCATCGTCCTAGA-3') and H1363 (5'-GGGGAAGGGAGGTTGACCTG-3'), specific for the 4977 by common deletion region, require the use of the modified enzyme, Expand™ High Fidelity, due to the large fragment size being amplified and the specialised 'long' PCR program.

The amplified DNA fragments from the D-loop region or the common deletion region were analysed by polyacrylamide gel electrophoresis on a 5% 37:1 acrylamide: bisacrylamide gel.

Insemination of biopsied oocytes

The B1 to B8 oocytes and the C1 oocyte were fertilized by either ICSI or IVF according to standard protocols (1,2). The insemination was performed immediately after cytoplasmic biopsy of the oocyte.

RESULTS

Oocyte fate after cytoplasmic biopsy

Oocytes A1 to A5 were not visibly affected by the cytoplasmic biopsy procedure and showed no signs of degeneration at 48 hours after biopsy. Oocytes B1 to B3 and B5 to B8 showed no signs of degeneration at 48 hours after biopsy. Oocyte C1 showed no signs of degeneration at

48 hours after biopsy. A single oocyte, B4, degenerated at 17 hours after biopsy (Table 2).

Table 2

Oocyte	Cytoplasmic Biopsy	17 hour post biopsy	48 hour post biopsy
A1	YES		No degeneration
A2	YES		No degeneration
A3	YES		No degeneration
A4	YES		No degeneration
A5	YES		No degeneration
B1	YES		No degeneration
B2	YES		No degeneration
B3	YES		No degeneration
B4	YES	Degenerated	
B5	YES		No degeneration
B6	YES		No degeneration
B7	YES		No degeneration
B8	YES		No degeneration
C1	YES		No degeneration

PCR amplification of mitochondrial DNA from cytoplasmic biopsy

Consideration was given to the amplification of mitochondrial DNA from a cytoplasmic biopsy taken from an oocyte. It was necessary to calculate the approximate number of mitochondria that would probably be obtained in such a biopsy to determine whether there would be sufficient template DNA for the PCR.

The cytoplasmic biopsy was obtained by using an ICSI pipette as described above. It was estimated that the volume of this cytoplasmic biopsy would be approximately 8pl. As the volume of an oocyte is approximately 500pl, the cytoplasmic biopsy removed is estimated to comprise

approximately 2% of the cytoplasm. In a conservative estimate, there are approximately 100,000 mitochondrial genomes per oocyte (3). In accordance with this estimate, the biopsy would remove approximately 1000 mitochondria. This amount of mitochondria is within the amplification capabilities of the polymerase chain reaction.

PCR amplification from the D-loop region

A 400 bp D-loop fragment was amplified from the cytoplasmic biopsy of oocytes A1 to A5 with 35 cycles of amplification (Figure 1).

PCR amplification from the common deletion region

A 5.5kb fragment was amplified from the cytoplasmic biopsy of oocytes B1 to B3 and B5 to B8 (data not shown).

Insemination of biopsied oocytes

The results of the insemination of the biopsied oocytes, B1 to B8 and C1 are shown in Table 3.

Table 3

Oocyte	IVF/ICSI	17 hour post biopsy	48 hour post biopsy
B1	ICSI	2PN	2 cell
B2	ICSI	2PN	No division
B3	ICSI	3PN	2 cell
B4	ICSI	Degenerated	
B5	IVF	Not fertilized	
B6	IVF	Not fertilized	
B7	IVF	Not fertilized	
B8	IVF	Not fertilized	
C1	ICSI	2PN	2 cell

PN= pronuclei observed

All oocytes that were subject to the ICSI protocol, (except B4) were fertilized. Two pro-nuclei were observed in B1, B3 and C1, and 3 pro-nuclei were observed in B3 at 17 hours after fertilization. The oocytes B1, B3 and C1

divided and progressed to the 2 cell stage at 48 hours post fertilization. No cell division was observed in B2 at 48 hours.

Although the B5 to B8 oocytes had not degenerated at 48 hours post biopsy, none of these oocytes were fertilized by insemination via the IVF protocol.

DISCUSSION

Degeneration was observed in only one of the 14 oocytes which were subjected to the cytoplasmic biopsy technique. As some oocytes tend to degenerate at approximately 24 hours post retrieval (4,5), it is possible that the B4 oocyte degenerated independently of the cytoplasmic biopsy technique. It should be noted that the oocytes A1 to A5 were matured in a medium formulated specifically for insemination, rather than oocyte maturation.

The results show that the cytoplasmic biopsy technique can be generally applied up to 24 hours post retrieval of the oocyte. The biopsy technique is therefore able to be used together with known fertilization techniques, including for example, ICSI and IVF, which are generally used approximately 4 to 6 hours post retrieval of the oocyte. Although in the present study, insemination was performed immediately after biopsy, it is expected that the biopsy may be performed after insemination.

We have found that the percentage of oocytes that are morphologically intact after the ICSI procedure (i.e the injection of sperm without removal of cytoplasm) is 95.2% (based on 2649 oocytes injected) (unpublished results).

By way of comparison, the standard for other types of micro-manipulation was difficult to establish, with the rate of degeneration after polar body and underlying cytoplasm removal for nuclear transplantation in other

species not being documented (6, 7). A survival rate of 30.8% (67/217) quoted for foreign mitochondrial injection and a 6% lysis rate has been reported with ooplasmic injection in human oocytes.

Although removal of the cytoplasm is more invasive than ICSI alone, the overall oocyte survival rate in the present study is surprisingly higher than our above discussed survival rates after standard ICSI. Thus the cytoplasmic biopsy technique can be considered potentially acceptable clinically.

The amplification of fragments from the D loop region and the common deletion region of the mitochondrial genome from mitochondria in the cytoplasmic biopsy demonstrates that there is a sufficient source of template DNA in the biopsy sample for analysis by PCR, and that fragments of the order of from less than 0.5 kb to greater than 5 kb can be amplified from the sample. The amplification of fragments from 2 independent loci of the mitochondrial genome suggests that mutations at other loci of the mitochondrial genome, in particular the mutations described in Table 1, can be amplified from the cytoplasmic biopsy sample using PCR and specific oligonucleotides.

The fertilization rate and subsequent cleavage rate was encouraging considering the oocytes donated in these experimental cohorts were greater than 24 hours old at the time of cytoplasmic biopsy and attempted fertilization. Previous studies have indicated that culture for a period exceeding 20 hours before insemination can compromise oocyte fertilization and development (4,5). In addition, oocytes matured in vitro lack the capacity of oocytes matured *in vivo* to maintain a high rate of cleavage (8). For this reason, it was not surprising that the B5 to B8

oocytes were not fertilized subsequent to insemination via the IVF protocol.

2. Embryonic cells

Materials and Methods

Source of embryonic cells

Frozen research embryos (3 * two pronuclear embryos, 1 * 2 cell embryo and 1 * 4 cell embryo) were thawed and equilibrated in growth medium.

Isolation of embryonic cell cytoplasmic fraction

The 3 single cell embryos and 1 cell from each of the multi cell embryos were biopsied using a standard ICSI pipette. In each case the pipette was introduced into the cytoplasmic region and an aliquot of cytoplasm corresponding to approximately 5% of the cell volume was withdrawn. This aliquot was delivered into a sucrose solution. The embryos were returned to the incubator for continued growth.

Analysis of mitochondrial genome in embryonic cell cytoplasmic fraction

The preparations of cytoplasm were made alkaline with Potassium Hydroxide and heated to 90°C for 5 minutes. After neutralizing with TrisHCI an aliquot was added to a reaction mix containing mitochondrial DNA specific primers designed to amplify a 271 base pair fragment corresponding to mtDNA positions 8201 and 8472. This region of the mitochondrial genome comprises part of the coding region for an oxidase and an ATPase. The mix was PCR amplified using a thermal cycler capable of real time monitoring of increasing fluorescence associated with the amplification of double stranded DNA. Reagent and medium blanks were negative for any fluorescence changes. Analysis of DNA

copy number was by comparison to an external dilution of control human DNA.

RESULTS

PCR amplification of mitochondrial DNA from cytoplasmic biopsy

All five samples showed the presence of mitochondrial target DNA. Table 4 shows estimated mtDNA copies in biopsy samples

Table 4

Embryo type	Mt DNA copy number
2PN	2,040
2PN	4,456
2PN	15,304
2 Cell	15,388
4 Cell	10,676

Embryo fate after biopsy

Approximately 24 hours after the biopsy procedure the embryos were examined using microscopy to ascertain their individual continued viability. Only 1 of the 2PN embryos had failed to divide although some degenerating cell material was observed in the original 2 cell and 4 cell embryos. Three days later, further observations showed two embryos had progressed to blastocyst formation.

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